

# A Complex between Prostate-specific Antigen and $\alpha_1$ -Antichymotrypsin Is the Major Form of Prostate-specific Antigen in Serum of Patients with Prostatic Cancer: Assay of the Complex Improves Clinical Sensitivity for Cancer<sup>1</sup>

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## ABSTRACT

We have studied the forms of prostate-specific antigen (PSA) in serum of patients with prostatic cancer and benign prostatic hyperplasia. Fractionation of serum by gel filtration and assay of the fractions for PSA showed that a considerable part of the PSA immunoreactivity in serum consisted of complexes that were larger than PSA. The complexes were assayed by time-resolved immunofluorometric assays based on an antibody against PSA on the solid phase and europium-labeled antibodies against various protease inhibitors as indicator antibodies. In addition to its monomeric form, PSA was found to occur in complex with  $\alpha_1$ -antichymotrypsin. The proportion of the  $\alpha_1$ -antichymotrypsin complex was a major form of PSA and it increased with increasing PSA concentrations, being over 85% at PSA levels exceeding 1000  $\mu\text{g/liter}$ . A complex with  $\alpha_1$ -protease inhibitor was also observed in serum of patients with prostatic cancer and very high levels of PSA. Complexes with  $\alpha_2$ -macroglobulin and inter- $\alpha$ -trypsin inhibitor were detected, but their concentrations were low and similar in sera of cancer patients, normal men, and normal women, suggesting that they were not prostate derived. Commercial immunoradiometric assays for PSA were found to measure free PSA and its complexes with  $\alpha_1$ -antichymotrypsin but not the complexes with  $\alpha_2$ -macroglobulin and inter- $\alpha$ -trypsin inhibitor. The proportion of the PSA- $\alpha_1$ -antichymotrypsin complex was higher in patients with prostatic cancer than in those with benign hyperplasia. Therefore, assay of the complex had a higher sensitivity for cancer than assay of total PSA immunoreactivity.

## INTRODUCTION

PSA<sup>3</sup> is a  $M_r$  30,000 kallikrein-like protease occurring in the prostate and in seminal plasma (1, 2). PSA is a major protein in seminal plasma and it is responsible for the liquefaction of the clot formed immediately after ejaculation (3, 4).

PSA in serum is measured immunochemically by either competitive radioimmunoassay (5) or IRMA (6). In serum from healthy males the concentrations of PSA are below 2.8  $\mu\text{g/liter}$  (6, 7). Highly elevated levels of PSA occur in serum from patients with prostatic cancer, but elevated levels may also occur in connection with prostatic hyperplasia and other benign urological disorders (8, 9).

Little is known about the forms of PSA circulating in plasma. We recently found two serum samples with high levels of PSA, which gave false low results in a commercial IRMA (10). False

low results, also called the "hook effect," are observed in one-step immunometric assays at high antigen concentrations (11). However, the false low results also occurred with two-step incubation; therefore they were not explained by the hook effect (10). Fractionation of the sera by gel filtration revealed the presence of two components with different molecular size, but the components reacted equally in the PSA IRMA. Thus they did not explain the hook effect (10). However, if there were other PSA complexes, which bound to the solid phase antibody but not with the labeled antibody, they could cause a hook effect even in a two-step assay.

We have now further characterized the PSA immunoreactivity in serum by fractionating samples by gel filtration and measuring PSA immunoreactivity in the fractions. Because PSA is a protease, we assumed that it could form complexes with protease inhibitors. We therefore developed two-site time-resolved IFMAs for various PSA-inhibitor complexes. In each assay an antibody against PSA was adsorbed to the solid phase. As indicator antibody we used the IgG fraction of antiserum against the following serine protease inhibitors: ACT, IAT1, A2M, and API.

## MATERIALS AND METHODS

**Samples.** Serum samples were obtained from 67 patients with prostatic cancer, 30 with benign prostatic hyperplasia, 10 healthy male controls, and 12 female controls. Of the patients 8 were of T<sub>0</sub>, 14 of T<sub>1</sub>, 35 of T<sub>2</sub>, and 10 of T<sub>3</sub> according to the tumor-nodes-metastasis classification of the International Union Against Cancer. All patient samples were taken before initiation of therapy. Two of the samples have been briefly described before (10). All samples were stored at -20°C until assayed.

**Antisera and Commercial Assays for PSA.** Rabbit antisera against PSA, the serine protease inhibitors ACT, API, A2M, and IAT1, were from Dakopatts (Glostrup, Denmark). Cross-reactivity of the antisera was studied by immunodiffusion in 1% agarose gel. Assay kits for PSA (TANDEM-R-PSA) were obtained from Hybritech Europe S.A. (Liege, Belgium). This IRMA utilizes two monoclonal antibodies. For comparison we also used an IRMA (Coat-a-tube PSA) from Diagnostic Products Corporation (Los Angeles, CA).

**Buffers.** The buffer used in the gel chromatography experiments was TBS. The assay buffer in the IFMAs was TBS containing 5 g/liter bovine serum albumin and 0.15 g/liter bovine globulin. The wash solution contained 0.15 M NaCl, 0.5 g/liter NaN<sub>3</sub>, and 0.2 g/liter Tween 20.

**Labeling with Europium.** For labeling with europium, the immunoglobulin fraction was partially purified by precipitating the antiserum with 18% Na<sub>2</sub>SO<sub>4</sub>. The precipitate was dissolved in 0.1 M sodium carbonate, pH 9.3, and labeled with N<sub>3</sub>-DTTA-europium chelate as described before (12). Briefly 0.3 mg of chelate was added to 1 mg of IgG in 0.1 M NaHCO<sub>3</sub> buffer, pH 9.0. After incubation for 24 h labeled IgG was separated from excess chelate by gel filtration on a 1 x 45-cm Sephacryl S-200 column in TBS buffer. About 3-10 mol of europium was incorporated per mol of IgG. The concentration of IgG was estimated on the basis of the absorbance at 280 nm using a specific

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<sup>3</sup> The abbreviations used are: PSA, prostate specific antigen; ACT,  $\alpha_1$ -antichymotrypsin; API,  $\alpha_1$ -protease inhibitor; A2M,  $\alpha_2$ -macroglobulin; IAT1, inter- $\alpha$ -trypsin inhibitor; IFMA, immunofluorometric assay; IRMA, immunoradiometric assay; TBS, 0.05 M Tris-HCl, pH 7.7, containing 0.15 M NaCl and 7.7 mM NaN<sub>3</sub>; DTTA, N<sup>3</sup>-(p-isothiocyanatobenzyl)-diethylenetriamine-N<sup>1</sup>,N<sup>2</sup>,N<sup>2</sup>-tetraacetic acid.

absorbance of 1.3 for an immunoglobulin solution containing 1 g/liter. The labeled immunoglobulin was diluted in assay buffer to contain about 50 ng of labeled immunoglobulin in 200  $\mu$ l, which was the amount of label added to each well in the various complex assays. This amount of antibody had a fluorescence of  $5-15 \times 10^4$  cps.

**Time-resolved Immunofluorometric Assays for PSA and PSA Complexes.** The immunoglobulin fraction of the PSA antiserum was diluted in TBS to a protein concentration of 5 mg/liter and 200  $\mu$ l of this solution was pipetted into microtitration strip wells (Eflab, Helsinki, Finland) and allowed to adsorb to the solid phase for 20 h at 20°C. Unadsorbed antibodies were discarded and the nonspecific binding sites of the wells were saturated with TBS containing 10 g/liter of bovine serum albumin (Sigma). After incubation for 3 h the wells were emptied and stored in a moist chamber at 4°C. By using combinations of the PSA antibodies together with each of the protease inhibitor antibodies as tracers, the various PSA-inhibitor complexes could be measured specifically. To measure PSA we also used an IFMA with the same PSA antibodies on the solid phase and as tracer.

For assay of serum 25  $\mu$ l of sample and 200  $\mu$ l of assay buffer were pipetted into the wells. For assay of chromatographic fractions a sample volume of 200  $\mu$ l was used. After incubation for 1 h the wells were emptied, washed twice with wash solution, and filled with 200  $\mu$ l of europium-labeled antibody solution. After further incubation for 0.5 h the wells were emptied and washed four times with wash solution using an automatic washer (Delfia Platemash 1296-024; Pharmacia-Wallac, Turku, Finland). Enhancement solution (200  $\mu$ l) was added to the wells and after 5 min the fluorescence was measured for 1 s in a time-resolved Arcus 1230 fluorometer (Pharmacia-Wallac). The fluorescence was expressed as cps. Standard curves were constructed by using the standards of the Hybritech assay. The standards covered the concentration range 2–100  $\mu$ g/liter. In the PSA-ACT assay 25  $\mu$ l of the Hybritech standard diluted to contain 1  $\mu$ g/liter gave a net fluorescence of 2394 cps. For assay of all PSA-protease inhibitor complexes we expressed the concentrations in arbitrary units, 1 unit/liter corresponding to this fluorescence. The sensitivity of the assays was calculated on the basis of the mean fluorescence  $\pm$  2 SD of 10 samples containing assay buffer only (13).

**Gel Filtration.** Fractionation of serum samples was performed by gel filtration using a 1.6 x 70-cm column of Sephacryl S-300 (Pharmacia) and with TBS for elution. Flow rate was 15 ml/h and fractions of 1 ml volume were collected. The column was calibrated using IgG, bovine serum albumin, ovalbumin, and soybean trypsin inhibitor as molecular size markers.

## RESULTS

The IFMA for PSA had a sensitivity of 0.1  $\mu$ g/liter and an assay range up to 100  $\mu$ g/liter when the Hybritech standard was used (Fig. 1). The intra- and interassay coefficients of variation of the assay were 5–8 and 7–12% in the concentration range

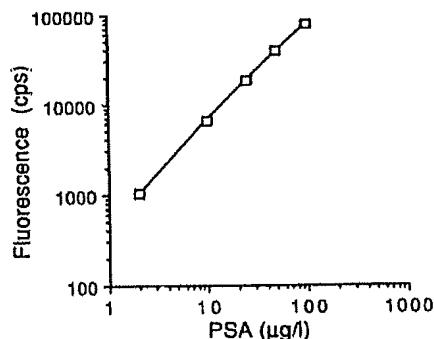


Fig. 1. Dose-response curve for time-resolved immunofluorometric assay of PSA. The PSA standards of the Hybritech kit were used. The background of about 1000 cps has been subtracted.

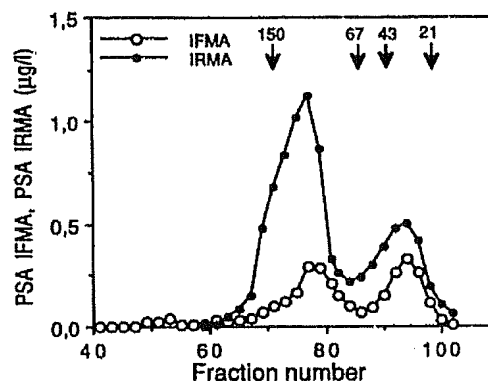


Fig. 2. Characterization of the Hybritech PSA standard by gel filtration and quantitation of PSA in the fractions by IFMA and by the Hybritech IRMA. Arrows, elution position of molecular weight standards.

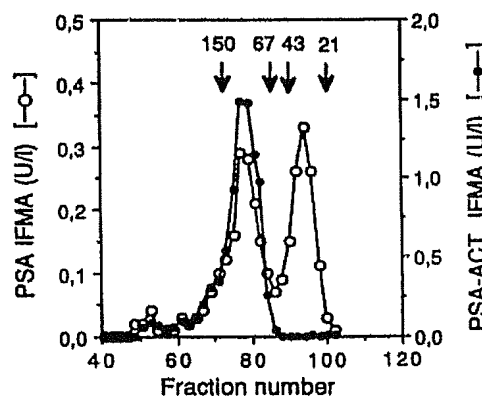


Fig. 3. Characterization of the PSA- $\alpha_1$ -antichymotrypsin complex in Hybritech PSA standard by gel filtration. The elution of PSA immunoreactivity is shown for comparison.

2–100  $\mu$ g/liter. Because the determination is performed as a two-step assay, it is not sensitive to interference by abnormal sample components, e.g., hemolysis or lipemia. The sensitivity of the Hybritech IRMA was about 1.8  $\mu$ g/liter. Comparison of the results for patient sera obtained by the PSA IFMA ( $y$ ) and the Hybritech assay ( $x$ ) showed that the correlation at PSA levels between 10 and 10,000  $\mu$ g/liter was good ( $r = 0.99$ ,  $y = 15.8 + 0.25x$ ) but the correlation was clearly lower at PSA levels below 10  $\mu$ g/liter ( $r = 0.86$ ,  $y = 0.19 + 0.81x$ ). At levels below 10  $\mu$ g/liter the IFMA measured 81% of the levels obtained by the Hybritech IRMA but at higher levels only 25%. Fractionation of the standard by gel filtration and assay of the fractions by both IRMA and the PSA IFMA revealed components with apparent molecular weights of about 100,000 and 30,000 (Fig. 2). The response of the  $M_r$  100,000 component was much smaller in the IFMA than in the Hybritech assay. As measured by the Hybritech IRMA about 70% of the immunoreactivity in the standard consisted of the  $M_r$  100,000 component (Fig. 2).

The assay for the PSA-ACT complex gave a very strong response with the Hybritech PSA standard. Assay of the fractions obtained by gel filtration of the standard showed that this immunoreactivity was associated with the  $M_r$  100,000 component. The PSA-ACT assay did not measure the  $M_r$  30,000 component (Fig. 3). In spite of this we used the Hybritech

standard as a provisional standard for the PSA-ACT assay. The sensitivity of the assay calculated on the basis of this standard was 0.2  $\mu\text{g/liter}$  corresponding to 0.2 unit/liter in the provisional units used. The intra- and interassay coefficients of variation of the assay were 6–9 and 8–12% in the concentration range 2–100  $\mu\text{g/liter}$ . Comparison of results obtained for cancer patient sera by the PSA-ACT method with those obtained by IRMA showed excellent correlation ( $r = 1.00$ ) and the levels were similar;  $y$  (PSA-ACT) =  $14.59 + 0.95x$  (PSA-IRMA). However, at low levels the correlation was not as good as at high levels (Fig. 4).

When serum samples from men with various levels of PSA (10–10,000  $\mu\text{g/liter}$ ) were fractionated by gel filtration, two components corresponding to PSA and PSA-ACT were also observed (Fig. 5). In samples with high PSA-levels the PSA-ACT complex dominated. In female sera these components were not seen (not shown). The proportion of PSA-ACT of total PSA immunoreactivity increased with increasing PSA levels (Fig. 6). In sera from patients with benign prostatic hyperplasia the mean proportion ( $\pm\text{SD}$ ) was  $51.4 \pm 9.2\%$  and the range was 41–59%. In cancer patients with the same range of PSA levels (1–28  $\mu\text{g/liter}$ ) the mean proportion was  $65.3 \pm$

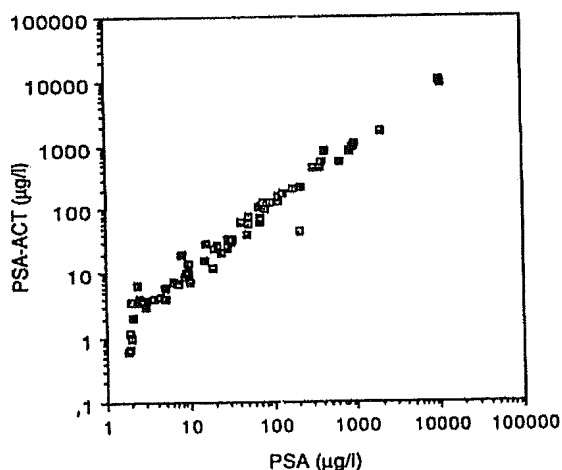


Fig. 4. Comparison of PSA concentrations in sera from patients with prostatic cancer measured by IRMA and the PSA-ACT complex concentrations measured by IFMA ( $n = 67$ ).

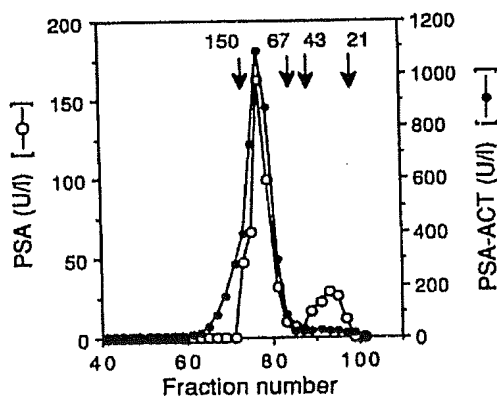


Fig. 5. Characterization of PSA immunoreactivity in a serum sample with a PSA level of 10,000  $\mu\text{g/liter}$  by gel filtration. PSA and the PSA-ACT complex were measured by IFMA.

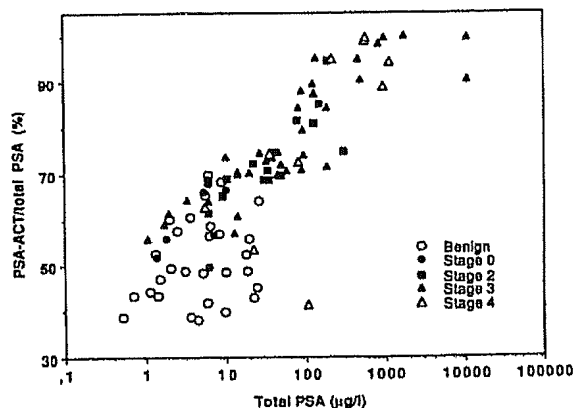


Fig. 6. Proportion of PSA-ACT complex of total PSA immunoreactivity in sera of patients with prostatic cancer as a function of the PSA concentration. The concentrations of PSA and PSA-ACT were measured by IFMA. The value for total PSA was calculated from the concentrations of PSA-ACT and PSA after correction of the PSA level for cross-reaction with PSA-ACT (21%).

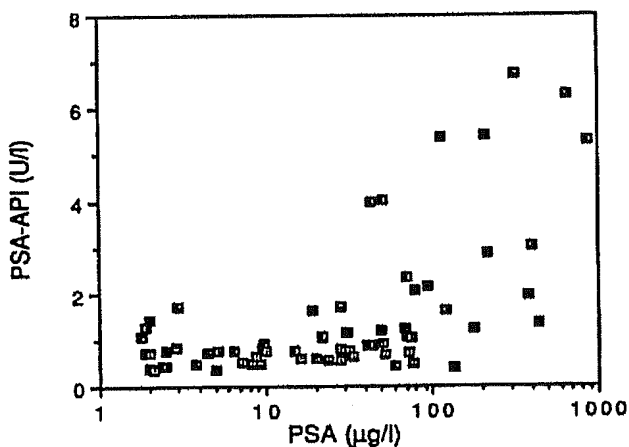


Fig. 7. Concentration of the PSA-API complex measured by IFMA as a function of the PSA concentration measured by IRMA in sera from patients with prostatic cancer.

9.2% and the range was 50–75%. The difference between these groups was highly significant ( $P < 0.001$ ) as estimated by Students'  $t$  test. In the whole group of cancer patients the mean proportion was  $74.8 \pm 14.1$  and the range was 41–100% (Fig. 6). In samples from healthy subjects the concentrations of PSA-ACT were close to the detection limit. Therefore it was not possible to calculate the proportion in these.

The main complex of PSA was that with ACT and clearly elevated levels of the PSA-API complex occurred only in samples with PSA levels over 40  $\mu\text{g/liter}$ . In these the levels tended to increase with increasing levels of PSA (Fig. 7).

In the two samples with very high PSA levels (10,000 and 9,000  $\mu\text{g/liter}$ ) that had given a false low result in the Hybritech assay, 75 and 98% of the total PSA immunoreactivity consisted of the PSA-ACT complex. These samples also contained a PSA-API complex at a concentration estimated to be less than 1% of that of the PSA-ACT complex. Gel filtration showed that the PSA-API complex eluted as two components with apparent molecular weights of about 190,000 and 80,000, respectively (Fig. 8). In addition the sample contained small amounts of an IATI complex with a molecular weight of about

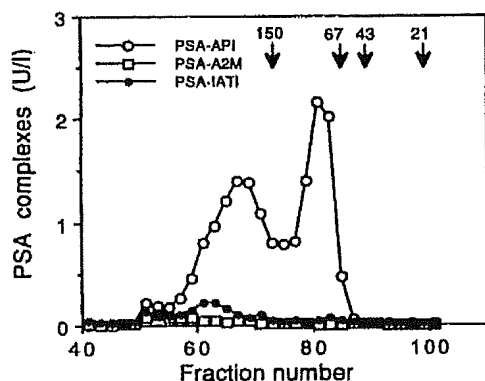


Fig. 8. Characterization of PSA immunoreactivity in a serum sample with a PSA level of 10,000  $\mu\text{g/liter}$  by gel chromatography. The fractions were assayed for their content of PSA-API, PSA-A2M, and PSA-IATI by IFMA.

Table 1 Clinical sensitivity for diagnosis of prostatic cancer of the various PSA assays

The specificity levels were determined on the basis of the results observed in 30 patients with benign prostatic hyperplasia, e.g., at a specificity level of 90%, 10% of the patients with benign prostatic hyperplasia had elevated levels of the marker. The cutoff levels giving a certain specificity are given in parentheses.

Specificity (%)	PSA (Hybritech)		PSA-ACT complex		Proportion of PSA-ACT	
	Cutoff ( $\mu\text{g/liter}$ )	Sensitivity (%)	Cutoff (U/liter)	Sensitivity (%)	Cutoff (%)	Sensitivity (%)
90	(16.5)	61	(10.0)	66	(65)	78
80	(12.5)	63	(7.6)	70	(59)	88
70	(8.4)	70	(4.5)	79	(57)	90

250,000 and an A2M complex eluting as a still larger complex (Fig. 8). However, the IATI and A2M complexes occurred at similar concentrations also in the Hybritech standard, in male samples with low levels of PSA, and even in female sera (not shown). When the antisera against serum proteinase inhibitors were subjected to immunodiffusion against normal serum, each antiserum gave a single precipitin line with no cross-reaction of the antibodies with each other (results not shown). This confirms the results from gel filtration showing that the polyclonal antisera specifically identified different complexes.

The IATI and A2M complexes were not recognized by the Hybritech PSA IRMA and the PSA IFMA. The PSA-API complex was not completely resolved from the PSA-ACT peak, thus it was not possible to evaluate whether the Hybritech assay recognized it.

In an attempt to further characterize the hook effect observed in two of the samples with the Hybritech IRMA we also assayed them with the PSA-IRMA from DPC. Undiluted samples gave a hook effect also in this assay both with one-step and two-step incubation. After dilution of the samples 100-fold, this assay gave the same results as the Hybritech assay.

The clinical specificity and sensitivity of PSA and PSA-ACT were evaluated by comparing the levels in patients with prostatic cancer and benign hyperplasia. The sensitivity for cancer was studied at levels giving 10–30% falsely elevated levels in patients with benign hyperplasia. This analysis showed that PSA-ACT had a clearly higher sensitivity at the specificity levels studied. The proportion of PSA-ACT/total PSA had a still higher sensitivity (Table 1). Conversely, at a sensitivity level of 70%, there were 9 “falsely” elevated PSA values, 6 PSA-ACT values, and only 3 proportion values in patients with benign prostatic hyperplasia.

## DISCUSSION

This study demonstrates that a major part of PSA in serum of patients with prostatic cancer occurs as a  $M_r$  100,000 complex between PSA and  $\alpha_1$ -antichymotrypsin. The proportion of the PSA-ACT complex increases with increasing PSA level. Patients with prostatic cancer have a significantly higher proportion of PSA-ACT than those with benign prostatic hyperplasia. Therefore determination of PSA-ACT and its proportion of total PSA has a better sensitivity for cancer than the conventional PSA assay measuring total PSA immunoreactivity. At a level giving a sensitivity for cancer of 70%, the number of falsely elevated results in benign hyperplasia is reduced by two-thirds, when the proportion of the complex is measured rather than PSA. The results were not dependent on the treatment, as all samples used in this study were taken before initiation of therapy. The improved differentiation between prostatic cancer and hyperplasia would mean a substantial improvement of the clinical utility of the PSA assay, if our results can be confirmed in a larger study.

In seminal plasma, PSA occurs as a  $M_r$  30,000 protein (14). Because PSA is a kallikrein-like protease, complexes with the large excess of serine protease inhibitors in plasma may be expected to form, when this protease is released in active form into blood.  $\alpha_1$ -Antichymotrypsin seems to be the inhibitor with the highest affinity for PSA, inasmuch as the PSA complex with this inhibitor is the dominating form. When the PSA levels are very high, other complexes are also formed. This may be a spillover effect, and the complex appearing next was that with  $\alpha_1$ -protease inhibitor. Because free protease is not likely to occur in plasma in the presence of a huge excess of inhibitors, the  $M_r$  30,000 form of PSA could represent a proenzyme or an inactivated form of PSA. Inactivation could be caused by partial proteolysis. Part of the PSA isolated from seminal plasma actually occurs in a “nicked” form, the peptide bond at the carboxyl end of the lysyl residue at position 145 being cleaved (2).

Complexes with  $\alpha_2$ -macroglobulin and inter- $\alpha$ -trypsin inhibitor were also observed but the levels of these did not correlate with the PSA level and they even occurred in women. Thus they may represent inhibitor complexes with proteases related to PSA. Proteases in complex with  $\alpha_2$ -macroglobulin are rapidly removed from circulation (15). Thus PSA may form complexes with  $\alpha_2$ -macroglobulin even if they are not readily detectable in serum. It has been suggested that antibodies to PSA may react with kallikrein because of the considerable sequence homology between PSA and glandular kallikrein (16). Kallikrein has also been shown to exist in complex with protease inhibitors and the most effective kallikrein inhibitors in human plasma appear to be  $\alpha_2$ -macroglobulin and C1 inactivator, but complexes with  $\alpha_1$ -protease inhibitor can also form (17). Therefore it is possible that the polyclonal antiserum used in our sandwich assays reacts with such complexes. However, we cannot rule out the possibility that the fairly low levels observed were caused by nonspecific factors.

The PSA-inhibitor complexes with molecular weights above 100,000 were not measured by the Hybritech IRMA or by our IFMA based on polyclonal PSA antibodies. Such complexes could still react with the monoclonal antibody used on the solid phase in the IRMA causing partial saturation and a false low result even with two-step incubation. This could explain the earlier observed hook effect in the two samples with very high PSA levels (10). Circulating antibodies to PSA have been

described in patients with advanced prostatic cancer (18). Such antibodies could also form PSA complexes causing false results. Because the monoclonal antibodies used in the Hybritech assay were not available, we could not directly test these possibilities and the final explanation remains open. For comparison we assayed the samples giving a hook effect with another commercial immunometric assay for PSA and observed the same hook effect.

In PSA-inhibitor complexes part of the antigenic sites may be supposed to be covered, and in some complexes this effect is probably more pronounced. The polyclonal antibodies used in this study showed reduced binding to the PSA-ACT complex, and the other complexes were not measured at all by the PSA IFMA based on these antibodies. However, the antibodies recognized at least one PSA determinant in some of the other complexes as evidenced by the response obtained, when they were used in combination with antibodies against  $\alpha_1$ -protease inhibitor and  $\alpha_1$ -antichymotrypsin.

The Hybritech assay for PSA measured both the *M*, 30,000 and *M*, 100,000 forms of PSA, but because appropriate standards were not available, it was not possible to evaluate whether these components gave equimolar responses. The PSA IFMA based on polyclonal antibodies underestimated the *M*, 100,000 complex; therefore the levels of PSA measured in serum by this assay were clearly lower than those obtained by the Hybritech assay, when the same standard was used. The difference was more pronounced in samples with high PSA levels because these contained a higher proportion of complexes.

The presence of two components in the PSA standard reveals a potential problem with the standardization of this assay. The concentrations of PSA are expressed in weight units, but at least in the standard provided with the Hybritech kit, most of the immunoreactivity consists of a complex in which a major part by weight is  $\alpha_1$ -antichymotrypsin rather than PSA. It would be important to know how this preparation has been calibrated. In spite of the ambiguity of the standard, the two commercial PSA assays used gave very similar results, but other PSA assays have been found to give clearly higher results than the Hybritech assay (19). The problems with the standardization also concern the results obtained by the IFMA:s for PSA and PSA-ACT. Therefore the absolute levels measured by these assays may change, when appropriate standards become available. This would not affect the clinical performance of the PSA-ACT assay, because results for patients and controls will change in the same way.

In conclusion, our results show that a major part of PSA in

serum appears in complex with  $\alpha_1$ -antichymotrypsin, and the proportion of this complex is higher in patients with prostatic cancer than in those with benign prostatic hyperplasia. These findings suggest that assay of the complex and its proportion to total PSA immunoreactivity can be used to differentiate between PSA elevations caused by benign and malignant prostatic disease.

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